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A Novel Deletion Mutant of Hepatitis B Virus Surface Antigen

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HBsAg is the most important serological marker for acute or chronic hepatitis B. Nevertheless, there are reports of HBsAg-negative virus carriers, either with anti-HBc as the only marker for hepatitis B virus (HBV) infection or even positive for anti-HBs and anti-HBc. We report isolates from a patient, in which a deletion in the HBs-gene was associated with persisting viremia in the presence of anti-HBs. The 62-year-old female, infected most likely by her husband, had detectable markers of chronic active hepatitis B, such as HBsAg, HBeAg, and anti-HBc-IgM, for 2 years. The patient then seroconverted to anti-HBs, although HBeAg and anti-HBc-IgM remained detectable. At this time, semiquantitative polymerase chain reaction showed about 10^4 viral genomes per milliliter of serum. Direct sequencing of the amplified products revealed a major population of DNA molecules with a deletion of nucleotide 31 of the HBs-gene, which up to now has not been described. This deletion led to a frame-shift and introduced a stop-codon after 21 amino acids of the sHBsAg. We suspect that this deletion, and the resulting HBsAg lacking the major epitopes recognized by specific antibodies, could favor ongoing viral replication, despite the presence of anti-HBs. However, because the reading frame of the polymerase was also severely damaged by this deletion, it is assumed that a minor population of intact genomes was present to help in the formation of virus particles. ***J. Med. Virol. 58:105–110, 1999.***

KEY WORDS: serology; genetic variant; deletion mutant; direct sequencing; clonal analysis

INTRODUCTION

Serological evidence for acute or chronic hepatitis B is provided most commonly by assays detecting the small surface antigen (sHBsAg), which is the major of three co-terminally expressed glycoproteins of the viral envelope. Its detection is believed to prove the presence of hepatitis B virus (HBV) in the liver and the peripheral blood, whereas its absence excludes infectivity to a high degree of certainty.

Nevertheless, there are a number of reports of HBsAg-negative virus carriers and, even more importantly, of cases of post-transfusion type B hepatitis, despite these screening measures [Hoofnagle et al., 1978; Bréchot et al., 1985; Larsen et al., 1990; Sanchez-Quijano et al., 1993]. For example, sera of at least 10% (up to 40% in "high risk" groups) of individuals with antibodies to the hepatitis B core antigen (anti-HBc) as the only marker of HBV infection contain HBV-DNA as detected by sensitive polymerase chain reaction (PCR) assays [Kroes et al., 1991; Jilg et al., 1995]. A certain percentage of these cases is due to circulating immune complexes of HBsAg and anti-HBs, preventing both the antigen and the antibody from detection by routine methods [Ackerman et al., 1994; Joller-Jemelka et al., 1994]. Moreover, even cases of anti-HBs-positive carriers were described [Zhang et al., 1993; Penna et al., 1996; Rehermann et al., 1996], although antibodies against the viral envelope usually neutralize the virus and confer protection from infection.

A well-characterized explanation for the latter pattern is a mutation of amino acid 145 of the sHBsAg from glycine to arginine. This mutation was seen repeatedly in a child born to an HBV-positive mother and who was immunized actively and passively immediately after birth [Carman et al., 1990; Fujii et al., 1992; Waters et al., 1992; Oon et al., 1995; Nainan et al., 1997], and was also observed in liver transplanted HBV carriers who received hepatitis B immunoglobulin to prevent reinfection of the graft. The role that this variant may play in relapses in these patients remains a subject of contentious discussion [Brown et al., 1992; Schätzl et al., 1997; Protzer-Knolle et al., 1998].

In contrast to this and other rare variants found in

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the context of external selective pressure [Harrison et al., 1994; Howard et al., 1994], we present here a patient with prolonged viral persistence in the presence of spontaneously produced anti-HBs. In this case, a so-far undescribed deletion of a single nucleotide in the HBs-gene, leading to a premature translational stop, seems to be correlated with this uncommon situation.

METHODS

Serology

Serological tests for HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, and anti-HBc-IgM, as well as HBsAg confirmation assays, were carried out using commercially available standard microparticle enzyme immunoassay procedures (AxSym, Abbott Diagnostika, Wiesbaden, Germany), according to the manufacturer's instructions. Positive results for anti-HBc were confirmed utilizing the respective assay with a different system (IMx, Abbott Diagnostika).

PCR and Sequence Analysis

Viral nucleic acid was isolated from 400 μl of serum using the QIAamp blood kit (QIAgen, Hilden), eluted in 100 μl of ddH_2O, and either processed immediately or stored at −20°C. For the amplification of HBV-specific sequences, 10 μl of eluate were used. The primers and reaction conditions for amplifying and sequencing the HBs-gene were the same as described previously [Weinberger et al., 1997]. The assay was shown to be sensitive enough to detect approximately 50–100 copies of the viral genome per milliliter of serum. In addition to the analytical agarose gel electrophoresis, the identity of the amplificates was confirmed by Southern Blotting, using a digoxigenin-labeled 400-bp probe with alkaline phosphatase-coupled anti-digoxigenin (Boehringer, Mannheim, Germany) and CSPD bioluminescence substrate (Tropix, Bedford). Semiquantitative results were attained by comparing duplicates of a serial dilution of plasmid molecules (10^7 down to 1 molecule per reaction) containing a complete standard HBV genome in a pBR322 vector (plasmid pHBV991, Genbank accession number of the insert X51970). Appropriate measures were taken to avoid cross-contamination [Kwok and Higuchi, 1989]; their effectiveness was tested routinely by testing known negative serum samples and reagent controls. The sequence of the complete s-gene was obtained by forward and reverse reading of overlapping fragments using fluorescent gel or capillary sequencing systems (ABI 373A or 310, respectively, Applied Biosystems, Weiterstadt, and PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit, Perkin Elmer, Norwalk, CT). To identify mutations, the new genomic sequences were compared to databanks such as GenBank, EMBL, PIR or Swiss-Prot on the DNA, as well as the protein level. All sequence analyses were carried out by the appropriate components of the GCG Wisconsin package, version 9.1 (Genetics Computer Group, Madison, WI), using standard tables and algorithms, as suggested by the manufacturers.

Cleavage of Immune Complexes

To detect circulating immune complexes of HBsAg and anti-HBs, a protocol was used as described by Joller-Jemelka and colleagues [1994]. Briefly, the serum samples were incubated with 1 volume of 1.5 M glycine-HCl, pH 1.8 at 37°C for 2 hr, then 1 volume of 1.5 M Tris-HCl, pH 7.4 was added to neutralize the solution and prevent further acid proteolysis. After an additional 2 hr of incubation at 37°C, the samples were tested for the presence of free HBsAg in a commercial MEIA, as described above. Known negative sera from vaccinated individuals and known HBsAg-positive samples served as controls.

Particle Enrichment and Electron Microscopy

Viral and subviral particles were enriched from serum samples (typically 2–4 ml of serum diluted 1:2 with phosphate-buffered saline [PBS]) by ultracentrifugation at 70,000 × *g* for 18 hr at 4°C through a 20% (w/v) sucrose cushion and resuspended in 400 μl of PBS. Copper grids were floated on 80-μl drops of this suspension for 2 min at room temperature to let the particles adsorb to the surface. The grids were then washed on several drops of ddH_2O for 1 min each, air-dried for at least 1 hr and stained negatively with a 2% solution of phosphotungstic acid, pH 7.3 for 2 min, which yields better contrast and less artificial structures than the use of uranyl acetate, pH 3. After drying overnight, the grids were examined with a transmission electron microscope (Zeiss, Oberkochen) and details of interest were documented on 10-inch photographic plates.

RESULTS

A 62-year-old female developed acute hepatitis B in May 1994. In the process of investigating the source of infection, her husband was examined and found to be positive for HBsAg, HBeAg, and anti-HBc, as well as for anti-HCV (he was most likely infected during extensive surgical treatment and blood transfusions 2 years earlier). Within 4 weeks, the patient's transaminase values decreased and symptoms gradually disappeared; nevertheless, HBsAg and HBeAg persisted. After 1 year, she was still positive for HBsAg, HBeAg, and anti-HBc-IgM, the ALT level was slightly elevated (29 IU/l), and there was a moderate amount of HBV-DNA, corresponding to approximately 10^5 virus particles per milliliter of serum (Table I). Chronic persistent hepatitis B was diagnosed. However, during routine examination 7 months later, HBsAg could no longer be detected and anti-HBs was positive (17 IU/l), which was interpreted as the beginning of seroconversion and recovery, despite HBeAg and anti-HBc-IgM remaining positive. During the next 10 months HBsAg was not detected, anti-HBs levels increased from 17 to 124 IU/l, and HBeAg was still detected in addition to HBV-DNA, which was found repeatedly at a concentration corresponding to 10^3–10^4 virus particles per milliliter (Table II). Due to the obvious production of viral

TABLE 1. Case History

Date	Wife LS	Husband GS
08/1993		Surgical treatment (aortobifemoral bypass), administration of two doses of erythrocyte concentrate.
05/1994	Acute hepatitis B (GOT 374 U/l, GPT 693 U/l, γ-GT 286 U/l), AP 401 U/l, LDH 367 U/l, bilirubin 3.7 mg/dl.	
06/1994	Improvement after 4 weeks of hospital treatment (GOT 65 U/l, GPT 155 U/l, γ-GT 136 U/l), AP 179 U/l, bilirubin 1.35 mg/dl.	Serologically tested: positive for HBV (HBsAg, HBeAg, anti-HBc) and HCV (anti-HCV and confirmatory Western blot).
07/1995	Chronic persistent hepatitis B, low viremia and almost normal transaminases (GOT 15 U/l, GPT 29 U/l, γ-GT 15 U/l), bilirubin 0.5 mg/dl.	
Now	Resolved hepatitis B, still HCV negative.	Chronic active hepatitis B and C, moderate to high viremia of both viruses, moderate inflammatory activity, slightly raised transaminases (GOT 36 U/l, GPT 54 U/l), good general condition.

HBV, hepatitis B virus; HCV, hepatitis C virus.

TABLE II. Serological and PCR Data of Patient LS

Date	HBsAG	anti-HBs (IU/l)	anti-HBc	anti-HBc-IgM	HBeAg	anti-HBe	HBV-DNA
16.05.1994	+	–	+	+	+	–	ND
10./20.02.1995	+	–	+	–	+	–	10^5/ml
18.05.1995	+	ND	+	+	+	–	ND
12.07.1995	+	–	+	–	+	–	ND
05.10.1995	+	–	+	+	+	–	ND
22.02.1996	–	17	+	+	+	–	10^3/ml
01.08.1996	–	76	+	+	+	–	10^4/ml
Nov. 1996	–	124	+	+	+	–	10^4/ml
28.07.1997	–	>1000	+	–	–	+	–

PCR, polymerase chain reaction; HBV, hepatitis B virus; ND, not determined; +, positive; ±, limit value; –, negative.

particles in the presence of anti-HBs, a viral variant with changes in the HBsAg, possibly in or near the *a*-determinant, was suspected. Therefore HBV-DNA was extracted from the patient's serum and the s-gene was sequenced.

Surprisingly, a deletion of nucleotide 31 of the s-gene was found, which led to a frame shift introducing a stop codon after the first 21-nucleotide triplets of this gene (Fig. 1). Thus, only a truncated version of HBsAg containing 21 amino acids could be synthesized from this gene, which lacks the entire *a*-determinant. Identical sequencing results were obtained using HBV-DNA preparations from three different serum samples.

To confirm further this unexpected finding, as well as to determine whether this mutant was indeed the prevalent species, a pre-amplification clonal analysis technique was used: HBV-DNA isolated from serum was diluted down to a level at which theoretically only one copy could serve as the template for the amplification. PCR analysis of 30 diluted samples revealed products in 12 cases, which all showed the deletion of nucleotide 31. It can be concluded from this experiment, as well as from the initial results, which indicated no traces of nonmutated sequences, that the deletion mutant described above is highly predominant, making up more than 90% of all HBV-DNA present in the patient's circulation.

The presence of circulating immune complexes consisting of anti-HBs and wildtype HBsAg could be excluded. Three sequential samples of the patient's serum, which were all positive for HBV-DNA as well as anti-HBs, were examined following acid treatment to dissociate possible anti-HBs-HBsAg-complexes. No difference was observed between the patient's sera and six negative controls, whereas all initially HBsAg-positive samples decreased slightly in measured HBsAg concentration but remained clearly positive.

Electron microscopy of serum samples containing the mutated DNA revealed typical subviral particles with an average diameter of 17–21 nm. These particles could not be distinguished morphologically from those found in the serum of the patient's husband (whose HBV-DNA showed, apart from the deletion at nucleotide 31, an identical sequence of the s-gene, when compared with the wife's material; Fig. 1). Due to the relatively low level of viremia, only a few virions could be identified unambiguously in the samples; however, no apparent abnormalities with respect to diameter and morphology of the envelope and capsid could be observed. Interestingly, not a single filamentous particle was observed in the patient's material, although those structures were present in the expected proportion in the serum of her husband (Fig. 2).

The striking serological pattern of positive HBeAg

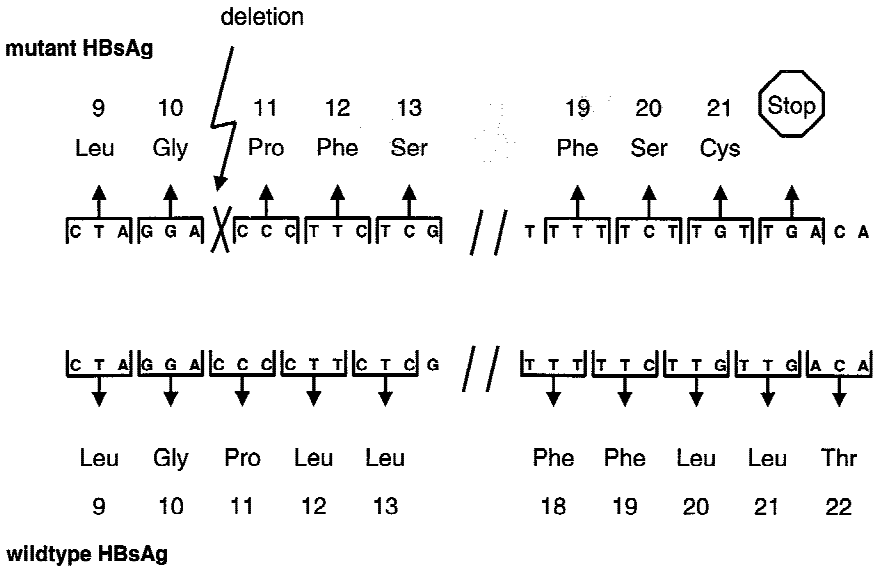

Fig. 1. Deletion mutant in hepatitis B virus (HBV) isolates from patient LS in comparison to the isolate found in her husband GS. Nucleotide 31 of the HBs-gene is deleted, introducing a frame-shift and leading to a premature stop-codon after 21 amino acids of the sHBsAg.

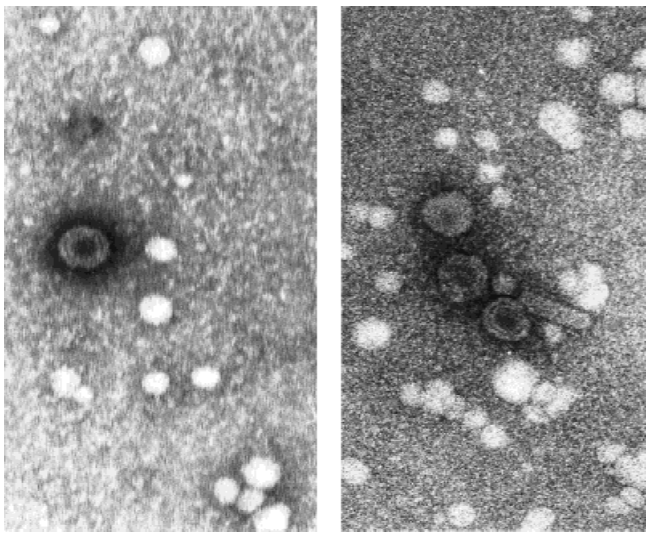

Fig. 2. Electron micrograph of viral and subviral hepatitis B virus (HBV) particles, as seen in the patient's (left panel) and her husband's (right panel) serum after negative staining with 2% phosphotungstic acid. No morphological differences or abnormalities could be observed; yet, there was an unusual lack of filamentous particles in the wife's samples. The scale bars represent 100 nm.

and HBV-DNA in the presence of anti-HBs and the predominance of the described deletion mutant was observed for almost 1 year (from February 1996 to November 1996). An examination in July 1997 revealed a substantial change: anti-HBs levels had increased significantly to values of more than 1,000 IU/l, HBeAg had disappeared, and HBV-DNA was no longer detectable, even after concentration of viral particles by ultracentrifugation was attempted. Thus, since the level of transaminases has also returned to normal levels, the patient now shows the typical pattern of resolved hepatitis B.

DISCUSSION

The findings presented here could suggest a relationship between the novel deletion mutant and the unusual serological pattern of HBV persisting in the presence of anti-HBs for almost 1 year. However, the nature of this relationship and the underlying molecular mechanisms are speculative. As shown schematically in Figure 3, the HBsAg predicted from our sequence data does not comprise those parts of the molecule that are exposed on the surface of the complete virions or the 22-nm particles. Thus, the *a*-determinant, which serves as the main target for the specific antibody response against the common subtypes of HBV, is missing, i.e., anti-HBs should not be able to bind to this mutated antigen. The assumption, however, that virus particles may exist that bear such truncated molecules on their surface—and contain the corresponding mutated DNA—raises serious questions regarding their structure, as well as the way they are produced.

Studies on certain artificial deletion mutants [Bruss and Ganem, 1991] suggest that such a drastically shortened HBsAg should not be able to form morphologically correct viral and subviral particles. Nevertheless, the nucleic acid that was detectable in the circulation could not be present as "naked" DNA in this amount, but rather had to be packaged in the complete HBV particles. In fact, a small number of virions were identified, in addition to 22-nm particles, by electron microscopy.

The lack of HBsAg could also be explained by the fact that during seroconversion, circulating immune complexes, consisting of "normal" HBsAg and anti-HBs, were present and prevented the antigen from being detected. This explanation could be excluded in this

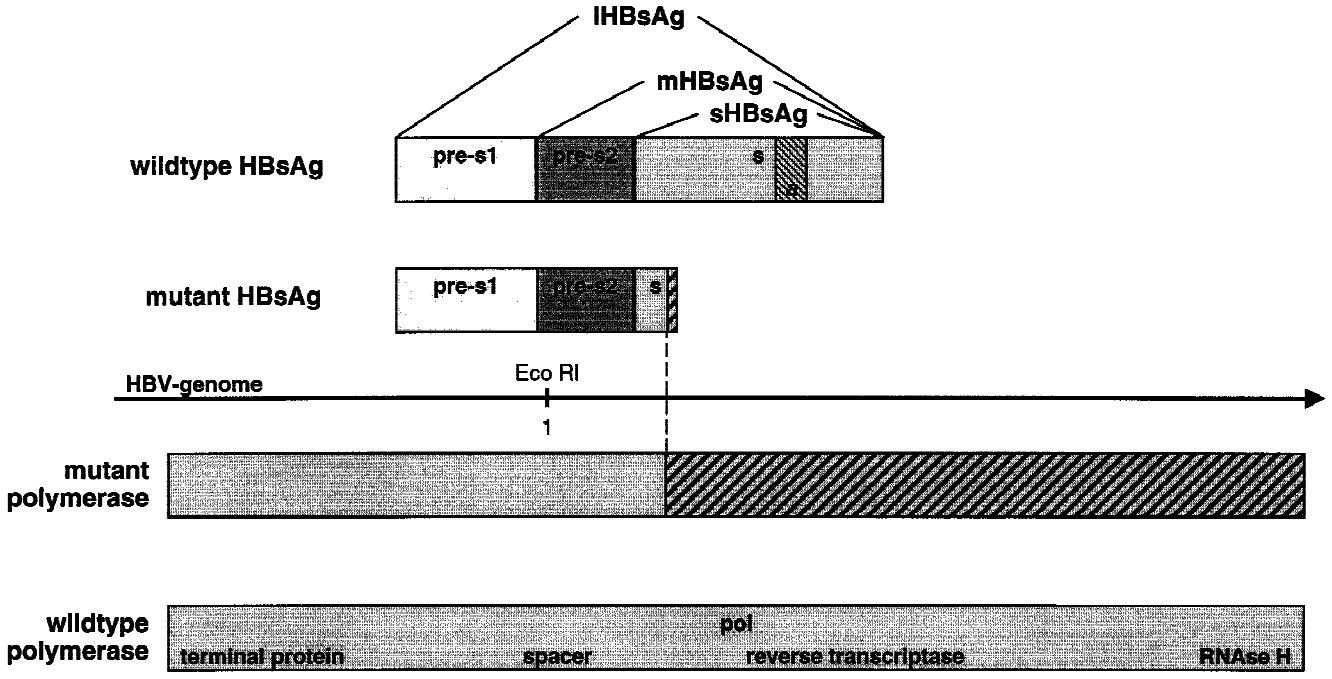

Fig. 3. Effects of the deletion HBs-Δ 31: HBsAg is drastically shortened, lacking the external parts including the group-specific *a*-determinant. More than half of the polymerase reading frame, particularly the reverse transcriptase domain, is also affected by the frame shift.

case because no serum sample revealed free HBsAg following acid cleavage, as described for solely anti-HBc-positive sera [Joller-Jemelka et al., 1994].

To address the functional aspect, it is indicated in Figure 3 that more than half of the polymerase reading frame, and especially most of the reverse transcriptase domain, are affected by the frame-shift mutation. This results in a protein that is no longer a properly functional polymerase. Yet, the parameters of active replication, such as anti-HBc-IgM and especially HBeAg, remained detectable. Therefore, it is assumed that the presence of a minor population of intact genomes provides help in the replication and the formation of intact virions. Because we could not detect "wild-type" genomes in the circulation, the complementation is most likely based on integrated viral DNA in the liver. Immune mechanisms, such as cytotoxic T-cells and cytokines killing hepatocytes that still produce viral proteins [Chisari, 1997] must be responsible for the elimination of the virus, as shown by negative PCR.

Theoretically, virus particles containing such truncated HBsAg molecules could represent true "escape mutants," because they would not bear a target structure for neutralizing antibodies, but possess the receptor binding pre-s-epitopes of the large or middle HBsAg. However, due to the lack of a functional polymerase, virions containing DNA with the described deletion would not be able to replicate in an HBV-naive host. Thus, it is unlikely that such mutants could pose a danger of infection for naive or, more importantly, immunized individuals.

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